# NOVEL LANTHIONINE ANTIBIOTIC COMPOSITIONS AND METHODS

## Related Applications

This application is a divisional of U.S. Patent Application Serial No. 10/047,676 filed January 14, 2002, now U.S. Patent No. 6,699,970. Application Serial No. 10/047,676 is a divisional of U.S. Patent Application Serial No. 09/627,376 filed July 28, 2000, now U.S. Patent No. 6,342,285.

## Grant Reference

The subject invention was made with government support under a grant from the National Institutes of Health (NIH RO 1 DE09082). The government has certain rights in the invention.

## Field of the Invention

The invention relates to polypeptide antibiotics and to the identification of genetic loci associated with expression of the antibiotics. The invention particularly describes a purified lanthionine-containing antimicrobial agent, DNA encoding the protein, and methods and compositions for treatments employing the antibiotic.

## Background of the Invention

Several species of bacteria inhabit the human oral cavity; among them *Streptococcus mutans* is considered a major etiologic agent responsible for dental caries. Loesche (1986) *Microbiol. Rev.* **50:**353-380. Previous studies showed a certain percentage of clinical isolates of *S. mutans* producing antimicrobial substances called mutacins. Caufield et al. (1985) *Infect. Immun.* **48:**51-56; Hamada et al. (1975) *Arch. Oral Biol.* **20:**641-648. Mutacins are

active against closely related species as well as a surprisingly wide spectrum of other Gram-positive bacteria. Parrot et al. (1990) *Can. J. Microbiol.* **36:**123-130. The ability to produce mutacins, combined with lactic acid production by *S. mutans* may contribute to the pathogenesis of these bacteria. Kleinberg, p. 605-624, in W.A. Nolte (ed.), Oral microbiology, The C.V. Mosby Company, St. Louis. Production of mutacins by *S. mutans* and other oral streptococci may also play a protective role to the host against pathogens such as Group A streptococci and *Streptococcus pneumoniae*. In this respect, mutacins may serve as antimicrobial agents in the future.

Lantibiotics are lanthionine-containing small peptide antibiotics that are produced by gram-positive bacteria. Jung (ed.), p. 1-34, in G. Jung and H.G. Sahl (ed.), Nisin and novel lantibiotics, ESCOM Sci. Publ., Leiden; Sahl et al. (1995) *Eur. J. Biochem.* **230:**827-853. The lantibiotics are ribosomally synthesized and post-translationally modified. The modification reactions include dehydration of serine and threonine residues and the addition of thiol groups from cycteine residues to the double bound to form lanthionines and β-methyllanthionines, respectively. Some dehydrated serine or threonine may remain as such in the mature lantibiotic molecule.

Based on the secondary structures, Jung assigned lantibiotics into two classes, Type-A (linear) and Type-B (globular). Jung (ed.), p. 1-34, in G. Jung and H.G. Sahl (ed.), Nisin and novel lantibiotics, ESCOM Sci. Publ., Leiden. de Vos et al. ((1995) *Molecular Microbiol.* **17:**427-437) and Sahl and Bierbaum (Sahl et al. (1998) *Annu. Rev. Microbiol.* **52:**41-79) further divided

each class into subgroups according to their primary peptide sequences. Thus, subgroup AI contains the nisin-like lantibiotics with nisin, subtilin, epidermin and pep5 as the most thoroughly characterized members. Allgaier et al. (1986) *Eur. J. Biochem.* **160:**9-22; Gross et al. (1968) *FEBS Lett.* **2:**61-64; Gross et al. (1971) *J. Am. Chem. Soc.* **93:**4634-4635; Kaletta et al. (1989) *Arch. Microbiol.* **152:**16-19; Weil et al. (1990) *Eur. J. Biochem.* **194:**217-223. Subgroup AII consists of lacticin 481, SA-FF22, salivaricin and variacin. Hynes et al. (1993) *Appl. Environ. Microbiol.* **59:**1969-1971; Piard et al. (1993) *J. Biol. Chem.* **268:**16361-16368; Pridmore et al. (1996) *Appl. Environ. Microbiol.* **62:**1799-1802; Ross et al. (1993) *Appl. Environ. Microbiol.* **59:**2014-2021. The genes responsible for the biosynthesis of the lantibiotics are organized in operon-like structures. The biosynthesis locus of all members in the subgroup AI lantibiotics consists of *lanA*, the structural gene for the lantibiotic; *lanB* and *lanC*, the modifying enzyme genes for post-translational modification of the preprolantibiotic; *lanP*, the protease gene for processing of the prelantibiotic; and *lanT*, the ABC transporter for secretion of the lantibiotic. In addition, epidermin and gallidermin have an extra gene, *lanD*, which is responsible for the C-terminal oxidative decarboxylation of the lantibiotic. Kupke et al. (1994) *J. Biol. Chem.* **269:**5653-5659; Kupke et al. (1995) *J. Biol. Chem.* **270:**11282-89. In comparison, subgroup AII lantibiotics have simpler genomic organizations. In subgroup AII, *lanB* and *lanC* are combined into one gene, *lanM*, and *lanP* and *lanT* are combined into *lanT*. Chen et al. (1999) *Appl. Environ. Microbiol.* **65:**1356-1360; Qi et al. (1999) *Appl. Environ. Microbiol.*

**65:**652-658; Rince et al. (1994) *Appl. Environ. Microbiol.* **60:**1652-1657. All lantibiotic loci also contain a set of immunity genes, which are responsible for self-protection of the producer strains. Saris et al. (1996) *Antonie van Leewenhoek* **69:**151-159. Moreover, the expression of the lantibiotic genes is usually regulated either by a single transcription regulator (Peschel et al. (1993) *Mol. Microbiol.* **9:**31-39; Qi et al. (1999) *Appl. Environ. Microbiol.* **65:**652-658) or by a two-component signal transduction system (de Ruyter et al. (1996) *J. Bacteriol.* **178:**3434-3439; Klein et al. (1993) *Appl. Environ. Microbiol.* **59:**296-303; Kuipers et al. (1995) *J. Biol. Chem.* **270:**27295-27304).

Previously, the isolation, biochemical and genetic characterizations of mutacin II, produced by a group II strain of the oral bacteria *Streptococcus mutans* was reported. Chen et al. (1999) *Appl. Environ. Microbiol.* **65:**1356-1360; Novak et al. (1994) *J. Bacteriol.* **176:**4316-4320; Novak et al. (1996) *Anal. Biochem.* **236:**358-360; Qi et al. (1999) *Appl. Environ. Microbiol.* **65:**652-658. Mutacin II belongs to subgroup AII in the lantibiotic family. Recently, the isolation and genetic characterization of mutacin III from the group III *S. mutans* strain UA787 was reported. Qi et al. (1999) *Appl. Environ. Microbiol.* **65:**3880-3887. The mature mutacin III is twenty-two amino acids in size, and shows striking similarity with another lantibiotic, epidermin, produced by *Staphylococcus epidermidis*. Allgaier et al. (1986) *Eur. J. Biochem.* **160:**9-22. The mutacin III biosynthesis gene locus consists of eight genes in the order of *mutR, -A, -A', -B, -C, -D, -P*, and *T*. The genomic organization and primary sequence of mutacin III places it in subgroup AI with

epidermin and gallidermin as its closest neighbors. Applicants disclosed herein the biochemical and genetic characterization of mutacin I. Comparison of the biosynthesis genes between mutacin I and mutacin III reveal striking similarities as well as important differences.

The cloning and sequencing of the novel mutacin I biosynthetic genes by using information from the conserved sequence derived from several other lantibiotics, and the isolation and purification of mutacin I is disclosed herein and provides a novel group of antibiotics which can be utilized as anti-microbial agents against, for example, presently antibiotic resistant microorganisms.

## Summary of the Invention

According to the present invention, an isolated and purified DNA sequence which encodes a lantibiotic, mutacin I, is disclosed. The nucleic acid sequence is set forth in SEQ ID No: 1 and the amino acid sequence is set forth in SEQ ID No: 2. Also disclosed are pharmaceutical compositions containing mutacin I and methods for their use.

## Brief Description of the Drawings

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended Figures. These Figures form a part of the specification. It is to be noted, however, that the appended Figures

illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figure 1. (A) The mutacin III biosynthesis genes. The orientation of the genes and their relative sizes are shown. *mutA* is the structural gene for prepromutacin I, and *mutA'* has no known function at present. *mutB* and -*C* encode the enzymes for dehydration and thioether bridge formation of premutacin I. *mutD* encodes a flavoprotein possibly responsible for oxidative decarboxylation of the C-terminal cycteine in premutacin I. *mutP* and -*T* code for the protease and ABC transporter, respectively, which are responsible for the processing and transportation of premutacin I. (B) Similarity between MutA and MutA'. The middle row shows the identical amino acids and the conserved changes (+). Arrowhead indicates the processing site in MutA. The leader peptide and the mature peptide moieties were determined based on MutA. (C) Effects of *mutA* and *mutA'* mutations on mutacin I production. Cells from an overnight culture plate were stabbed on TH agar plate and incubated at 37°C for twenty-four hours. The plate was heated at 80°C for one hour to kill the producing bacteria, then an overnight culture of the indicator strain NY101 was overlaid on top of the plate. The plate was inspected after an overnight incubation at 37°C.

Figure 2. Similarity between the mutacin I and mutacin III structural gene. The prepropeptides of mutacin I and mutacin III are compared using the sequence of preepidermin as a reference. The identical amino acids shared by all three lantibiotics are labeled with gray boxes, and the amino acids shared by

any of two lantibiotics are labeled with an open box. The conserved sequence FNLD, which is shared by all lantibiotics in subgroup AI (29) is underlined. Brackets indicate the pairs of amino acid residues involved with thioether bridge formation in epidermin (1).

Figure 3. Purification and EIMS analysis of mutacin I. (A) Elution profile of the first round purification of crude extract of mutacin I by reverse phase HPLC. One-ml fractions were collected along the course of elution and tested for antimicrobial activity (insert). (B) Elution profile of the second round purification using pooled fraction 6 from the first pass as starting material. Fractions 6 and 7 were active. (C) Electrospray ionization mass spectrometry (EIMS) of the purified mutacin I. The mass to charge ratio (m/z) for the doubly-charged molecule (1183) and the triply-charged molecule (788) are labeled. The estimated molecular weight was 2364 Da.

Figure 4. Biochemical characterization of mutacin I. (A) EIMS analysis of the ethanethiol-derivatized mutacin I. Peaks 1 and 2 are the doubly-charged molecule of 1791 Da and 1774 Da, respectively. The 1774-Da molecule may be a deaminated form of the 1792 Da molecule. Peak 3 may be a deaminated form of peak 4, both of which are singly charged. Peak 5 and peak 6 are triply-charged and doubly-charged molecule of 2719 Da, respectively. Peak 7 is a doubly-charged molecule of 2736 Da, which gives rise to the deaminated form of 2719 Da (peaks 5 and 6). Peak 8 is a singly-charged, deaminated form of peak 9, which has a molecular mass of 1793 Da. The expected molecular mass of mutacin I after insertion of six molecules of

ethanethiol is 2736 Da (2364 + 62x6), which correlated very well with the measured mass of 2736 as shown by peak 7. Addition of the two molecular masses of 1791 (peak 1) and 965 (peak 4) results in a molecular mass of 2756 Da, which would correlate well with the intact modified mutacin I of 2736 Da plus one molecule of $H_2O$ (from breakage of the molecule). (B) Proposed structure of mutacin I based on the data presented in (A) and in Fig. 2. Arrowhead indicates the position where the peptide bound is broken in the ethanethiol-modified mutacin I. The calculated molecular mass for each fragment is labeled. (C) EIMS analysis of mutacin III derivatized with ethanethiol under the same conditions as for mutacin I. The expected molecular mass for fully derived mutacin III is 2636 (see Table 1), and the measured molecular mass is 2638 from the doubly and triply charged peaks (peaks 2 and 3). The 2620-Da molecule as shown by peaks 1 and 4 are probably the deaminated form of the 2638-Da molecule. The 2576-Da molecule as shown in peak 5 resulted from addition of five molecules of ethanethiol (see Table 1).

**Detailed Description of the Invention**

The present invention provides an isolated and purified DNA sequence (SEQ ID No: 1) encoding for a novel lantibiotic, mutacin I, that has been isolated and characterized from *Streptococcus mutans* CH43.

Further, the present invention provides the isolated and purified DNA sequence for mutacin I designated as mutA (SEQ ID No: 1) and polymorphisms thereof specific for mutacin I.

By "isolated" it is meant separated from other nucleic acids found in bacteria. By "specific" is meant an isolated sequence which encodes the protein mutacin I.

Further, the present invention provides the amino acid sequence of the mutacin I structural protein SEQ ID No: 2, designated MutA and also referred to herein as mutacin I, functional variants thereof. The mutacin I protein has a molecular weight of approximately 2364 Da and is comprised of twenty-four amino acids in its mature form.

Modification to the nucleic acids of the present invention are also contemplated as long as the essential structure and function of the polypeptide encoded by the nucleic acids are maintained. Likewise, fragments used as primers or probes can have substitutions as long as enough complementary bases exist for selective, specific hybridization with high stringency.

Polymorphisms are variants in the gene sequence. They can be sequence shifts found between various bacterial strains and isolates which, while having a different sequence, produce functionally equivalent gene products. Polymorphisms also encompass variations which can be classified as alleles and/or mutations which can produce gene products which may have an altered function. Polymorphisms also encompass variations which can be classified as alleles and/or mutations which either produce no gene product, an inactive gene product, or increased levels of gene product.

The present invention also includes vectors including the mutacin I genes disposed therein. Such vectors are known or can be constructed by those

skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences. Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the nucleic acids in a different form. Phagemids are a specific example of such beneficial vectors because they can be used either as plasmids or as bacteriophage vectors. Examples of other vectors include viruses such as bacteriophages, baculoviruses, and retroviruses, DNA viruses, cosmids, plasmids, liposomes, and other recombination vectors. The vectors can also contain elements for use in either prokaryotic or eukaryotic host systems. One of ordinary skill in the art will know which host systems are compatible with a particular vector.

The vectors can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be found generally described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989, 1992) and Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989); Chang et al., *Somatic Gene Therapy*, CRC Press, Ann Arbor, Michigan (1995); Vega et al., *Gene Targeting*, CRC Press, Ann Arbor, Michigan (1995); *Vectors: A Survey of Molecular Cloning Vectors and Their Uses*, Butterworths, Boston, Massachusetts (1988); and Gilboa et al. (1986) and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. Introduction of nucleic acids by infection offer several advantages over other listed methods.

Higher efficiencies can be obtained due to their infectious nature. Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the vectors to specific cell types *in vivo* or within a tissue or mixed culture of cells. The viral vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

The above discussion provides a factual basis for the preparation and use of mutacin I. The methods used with and the utility of the present invention can be shown by the following non-restrictive examples and Figures.

DNA segments encoding a mutacin gene can be introduced into recombinant host cells and employed for expressing a mutacin I protein or peptide. The introduction of the mutacin I expressing DNA can be accomplished, for example, by the introduction of an organism transformed with the mutacin encoding DNA to act as a probiotic and produce the mutacin I *in situ* to protect against pathogens or other undesirable organisms. Alternatively, through the application of genetic engineering techniques, subportions or derivatives of selected mutacin I genes can be employed. Equally, through the application of site-directed mutagenesis techniques, one may re-engineer DNA segments of the present invention to alter the coding sequence, e.g., to introduce improvements to the antibiotic actions of the resultant protein or to test such mutants in order to examine their structure-function relationships at the molecular level. Where desired, one may also prepare fusion peptides, e.g., where the mutacin I coding regions are aligned

within the same expression unit with other proteins or peptides having desired functions, such as for immunodetection purposes (e.g., enzyme label coding regions).

**Pharmaceutical Compositions and Formulations**

Because of the broad spectrum of activity of mutacin I against a variety of microorganisms, mutacin I can be employed to treat multiple drug resistant bacteria such as certain strains of *S. aureus* which are known to be multiple drug resistant.

Pharmaceutical compositions comprising the disclosed mutacins may be orally administered, for example, with an inert diluent or with an assimilable edible carrier or they may be enclosed in hard or soft shell gelatin capsules or they may be compressed into tablets or may be incorporated directly with the food of the diet.

A therapeutically effective amount is an amount of mutacin I polypeptide, the pharmaceutically acceptable salts, esters, amides, and prodrugs thereof, that when administered to a patient or subject, ameliorates a symptom of the condition or disorder.

The compounds of the present invention can be administered to a patient either alone or as part of a pharmaceutical composition.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art.

Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

For oral prophylaxis, the polypeptide may be incorporated with excipients and used in the form of non-ingestible mouthwashes, dentifrices or chewing-type gums. A mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an antiseptic wash containing sodium borate, glycerin and potassium bicarbonate. The active ingredient may also be dispersed in dentifrices, including: gels, pastes, powders and slurries. The active ingredient may be added in a therapeutically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants.

The active compounds may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard or soft shell gelatin capsules, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The

percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of the unit. The amount of active compounds in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the following: a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compounds sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

The active compounds may also be administered parenterally, e.g., formulated for intravenous, intramuscular, or subcutaneous injection.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the

injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Dosage forms for topical administration of a compound of this invention include ointments, powders, sprays, and inhalants. The active component is admixed under sterile conditions with a physiologically acceptable carrier and any preservatives, buffers, or propellants as may be required. Ophthalmic formulations, eye ointments, powders, and solutions are also contemplated as being within the scope of this invention.

Intravascular devices, such as catheters, have become indispensable tools in the care of seriously ill patients. It is estimated that in the United States alone, 150 million catheters are purchased each year. However, due to the morbidity and mortality resulting from catheter-related infections and the high cost of managing such complications, the benefit derived from these

devices may be offset. It has been shown that bloodstream infection due to the use of intravascular catheters (IVC) increased dramatically during the last ten years. From 1975 to 1977, an estimated 3% infection occurred among IVC users, while in 1992 to 1993, this rate increased to 19%. The death rate from such infection is ~8000 to 16000 per year, exceeding the death rate for AIDS. The cost for treating IVC-related infections is ~132 to 1600 million per year.

Most infections came from the human skin and the hub of the catheter. Among the infectious bacteria, 40% are coagulase-negative staphylococci, such as *S. epidermidis*, and 14% are coagulase-positive *S. aureus*. The remaining are mostly other gram-positive bacteria such as bacilli and enterococci.

Prophylactic methods have been developed to prevent IVC-related infections. The first line of treatment is to sterilize the insertion site with iodine and 70% ethanol. However, compliance with the written protocol is low; only 23% operations follow the protocol. Another preventative measure is the use of catheters impregnated with antibiotics or antiseptic agents such as chlorhexidine and silver sulfadiazine. In clinical trials, mixed results were obtained using such catheters. In addition to the problem of drug resistance by the infecting bacteria, the antibiotics coating the catheters can also be washed away by body fluid, as the attachment of antibiotics to the catheter surface is mainly through ionic interactions.

Because of the urgency to solve the problem of IVC-related infection and the growing market for development of catheters resistant to bacterial attachment on the surface thereof, mutacins are an excellent choice for

prevention of IVC-related infections. Mutacin I has the following advantages over conventional antimicrobial agents: 1) it has a wide spectrum of antimicrobial activity against a wide range of gram-positive bacteria including the multidrug-resistant Staphylococci and Enterococci, the major culprits of IVC-related infections; 2) due to its unique mode of action against the sensitive bacteria, resistance to mutacin has not been observed; 3) mutacin is highly thermostable and works in a wide range of pH which makes it suitable for use in a wide range of conditions; 4) its hydrophobic nature can be advantageous for coating the surface of catheters and preventing adhesion of bacteria to the surface; and 5) because it is produced by a normal member of the human oral biota, it is unlikely to elicit immune response from the patient or has any toxicity to the host.

Active mutacin I compound can be coated onto intravascular devices and/or linked to polymers used in the manufacture of these devices to be used to prevent infection caused by intravascular devices. The mutacin I compounds of the present invention can be utilized alone in combination with at least one other entity, such as linked to a polymer, for the prevention or reduction of infection associated with a variety of medical devices such as indwelling tubes or catheters, artificial valves, pacemakers, implantable devices, etc., by incorporating, coating, or otherwise combining the active mutacin I compounds with the materials comprising the patient contact portions of the medical devices. The polymer can be a hydrophobic material or matrix that can be attached to an indwelling device such as a catheter through

hydrophobic bonding or can be tethered to the indwelling device through a molecular linker. The incorporation and/or combination of the active mutacin I compounds may be accomplished by coating the medical devices with active mutacin I compounds or by incorporating the active mutacin I compounds into the structure of the medical device. Because the mutacin I compounds of the present invention are very heat stable, they are able to withstand the conditions associated with their incorporation into the medical devices. By combining and/or incorporating the active mutacin I compounds of the present invention into medical devices, both active and passive infection control can be achieved at sites or for uses, which, in many instances, are highly susceptible or vulnerable to infection.

The term "pharmaceutically acceptable salts, esters, amides, and prodrugs" as used herein refers to those carboxylate salts, amino acid addition salts, esters, amides, and prodrugs of the compounds of the present invention which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of patients without undue toxicity, irritation, allergic response, and the like, commensurate with a reasonable benefit/risk ratio, and effective for their intended use, as well as the zwitterionic forms, where possible, of the compounds of the invention. The term "salts" refers to the relatively non-toxic, inorganic and organic acid addition salts of compounds of the present invention. These salts can be prepared *in situ* during the final isolation and purification of the compounds or by separately reacting the purified compound in its free base form with a suitable organic or inorganic

acid and isolating the salt thus formed. Representative salts include the hydrobromide, hydrochloride, sulfate, bisulfate, nitrate, acetate, oxalate, valerate, oleate, palmitate, stearate, laurate, borate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, naphthylate mesylate, glucoheptonate, lactobionate and laurylsulphonate salts, and the like. These may include cations based on the alkali and alkaline earth metals, such as sodium, lithium, potassium, calcium, magnesium, and the like, as well as non-toxic ammonium, quaternary ammonium and amine cations including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like. (See, for example, Barge et al., "Pharmaceutical Salts," *J. Pharm. Sci.*, 1977, 66:1-19 which is incorporated herein by reference.)

Examples of pharmaceutically acceptable, non-toxic esters of the compounds of this invention include $C_1$-$C_6$ alkyl esters wherein the alkyl group is a straight or branched chain. Acceptable esters also include $C_5$-$C_7$ cycloalkyl esters as well as arylalkyl esters such as, but not limited to benzyl. $C_1$-$C_4$ alkyl esters are preferred. Esters of the compounds of the present invention may be prepared according to conventional methods.

Examples of pharmaceutically acceptable, non-toxic amides of the compounds of this invention include amides derived from ammonia, primary $C_1$-$C_6$ alkyl amines and secondary $C_1$-$C_6$ dialkyl amines wherein the alkyl groups are straight or branched chain. In the case of secondary amines, the amine may also be in the form of a 5- or 6-membered heterocycle containing

one nitrogen atom. Amides derived from ammonia, $C_1$-$C_3$ alkyl primary amines, and $C_1$-$C_2$ dialkyl secondary amines are preferred. Amides of the compounds of the invention may be prepared according to conventional methods.

The term "prodrug" refers to compounds that are rapidly transformed *in vivo* to yield the parent compounds of the above formula, for example, by hydrolysis in blood. A thorough discussion is provided in T. Higuchi and V. Stella, "Pro-drugs as Novel Delivery Systems," Vol. 14 of the A.C.S. Symposium Series, and in *Bioreversible Carriers in Drug Design*, ed. Edward B. Roche, American Pharmaceutical Association and Pergamon Press, 1987, both of which are incorporated herein by reference.

In addition, the compounds of the present invention can exist in unsolvated as well as solvated forms with pharmaceutically acceptable solvents such as water, ethanol, and the like. In general, the solvated forms are considered equivalent to the unsolvated forms for the purposes of the present invention.

The compounds of the present invention can be administered to a patient at various dosage. For example, the dosage can depend on a number of factors including the requirements of the patient, the severity of the condition being treated, and the pharmacological activity of the compound being used. The determination of optimum dosages for a particular patient is well known to those skilled in the art.

## Examples

### Materials and Methods

**Bacterial strains and media.** The group I *S. mutans* strain CH43 originated from a Chinese school child as part of a natural history study of human caries. Strain CH43 contains a cryptic plasmid similar to other 5.6-kb plasmids within the *S. mutans* Group I strains. *S. sanguis* strain NY101 was used as the indicator for mutacin activity assays. CH43 and NY101 were grown on Todd-Hewitt (TH) plate with 1.6% agar (Difco Laboratories, Detroit, MI) unless indicated otherwise.

**Cloning and sequencing of the mutacin I biosynthetic genes.** Cloning and sequencing of the mutacin I biosynthesis genes were performed exactly as described previously. Qi et al. (1999) *Appl. Environ. Microbiol.* **65:** in press.

**Insertional inactivation.** The *mutA* and *mutA'* genes were inactivated separately by insertion of a kanamycin-resistant gene cassette exactly as described for mutacin III. Qi et al. (1999) *Appl. Environ. Microbiol.* **65:**625-658.

**Isolation and purification of mutacin I.** For mutacin production, CH43 was grown on TH/agar plate for one day under anaerobic conditions. The cells were then spread on a PHWP membrane with 0.3 μm pore size (Millipore Corp., Bedford, MA) on top of a TH plate containing 0.3% agarose. The plate was incubated at 37°C for two days anaerobically. The membrane was transferred to a new plate for continued incubation every two days, and the

old plate was frozen at -70°C. For mutacin isolation, the plates were thawed quickly in a 60°C water bath. The liquid medium was separated from the agarose debris by centrifugation and the supernatant was passed through a membrane with 0.45 μm pore size. Mutacin I was extracted with an equal volume of chloroform. Novak et al. (1994) *J. Bacteriol.* **176:**4316-4320. The precipitate was dried under a stream of air and washed once with double-distilled $H_2O$ ($ddH_2O$). The water-insoluble material (crude extract) was dissolved in 6 M urea and tested for antimicrobial activity by a plate assay after a serial dilution with $ddH_2O$. One arbitrary unit of activity (AU) was defined as the highest dilution that showed a clear zone of inhibition of the indicator strain NY101.

For purification, the crude extract of mutacin I was applied to a Source 15RPC column and eluted with a fragmented gradient A (0.1% TFA) and B (0.085% TFA in 60% acetonitrile) using a LKB Purifier (Amersham Pharmacia Biotech, Piscataway, NJ). The active fractions were pooled and dried in a lyophilizer. The pellet was redissolved in 0.25% TFA and subjected to a second round purification using a fragmented gradient of buffer A (0.1% TFA) and B (0.085% TFA in 80% methanol). The single active peak fraction was collected, dried in a lyophilizer, and used for sequence analysis and electrospray ionization mass spectrometry (EIMS).

**Chemical modification of mutacin I.** Fifty micrograms of purified mutacin I were dried under vacuum and resuspended in 90 μl of a derivatization mixture consisting of 280 μl ethanol, 200 μl water, 65 μl 5M

sodium hydroxide, and 60 µl ethanethiol as described). Meyer et al. (1994) *Anal. Biochem.* **223:**185-190. The reaction proceeded at 50°C for one hour under nitrogen, then stopped by the addition of 2 µl acetic acid. The reaction mixture was dried under vacuum and washed three times with 50% ethanol. The pellet was resuspended in 10 µl of 50% acetonitrile with 1% formic acid for EIMS analysis and peptide sequencing by Edman degradation.

**Nucleic Acid accession numbers:** The sequence for the mutacin I operon has been submitted to Genbank with the accession #AF207710 (AF267498), also designated SEQ ID No: 3.

## Results

**Cloning and sequencing of the mutacin I biosynthetic genes.** As described previously (Qi et al. (1999) *Appl. Environ. Microbiol.* **65:**652-658), while isolating mutacin III biosynthesis genes by PCR amplification using a pair of primers designed based on the conserved sequences among LanA and LanB proteins, the mutacin I biosynthesis genes were isolated using the same primers. Sequencing of the isolated PCR fragment demonstrated a striking similarity between the mutacin I and mutacin III genes. By chromosomal walking, the major part of the mutacin I biosynthesis operon was cloned and sequenced as shown in Figure 1A. It consists of eight genes in the order of *mutR, -A, -A', -B, -C, -D, -P,* and *-T,* which is possibly followed by the immunity gene *mutF* (SEQ ID No: 12). As in the mutacin III operon, MutR (SEQ ID No: 4) was the positive regulator for the expression of the mutacin I

operon (Qi et al. (1999) *Appl. Environ. Microbiol.* **65:**652-658. MutA (SEQ ID No: 5) and MutA' (SEQ ID No: 6) showed strong similarity to each other as shown in Figure 1B. Insertional inactivation of *mutA* and *mutA'* demonstrated that *mutA* was required for mutacin I production, while *mutA'* was not as shown in Figure 1C. This result suggested that, like *mutA* in the mutacin III operon, the *mutA* in the mutacin I operon was likely the structural gene encoding prepromutacin I. MutB (SEQ ID No: 7), -C (SEQ ID No: 8) and -D (SEQ ID No: 10) possibly constituted the modification apparatus for prepromutacin I, and MutT (SEQ ID No: 11) and -P (SEQ ID No: 10) are the ABC transporter and protease for transportation and processing of premutacin I, respectively. Other gene encoded mutacin I peptides include MutF (SEQ ID No: 12), MutE (SEQ ID No: 13), MutG (SEQ ID No: 14), OrfX (SEQ ID No: 15), OrfY (SEQ ID No: 16), and OrfZ (SEQ ID No: 17).

**Similarity between mutacin I and mutacin III biosynthesis genes.** The overall similarity between mutacin I and mutacin III biosynthesis genes was ~94% at the nucleotide level over the 10 kb operon. However, the differences between the two operons were not distributed evenly among the different genes. For example, from *mutR* to the region immediately upstream of *mutA*, the similarity was 99%, while in the *mutA* and *mutA'* coding regions, the similarity was only 89% and 91%, respectively. At the amino acid level, the two MutAs shared 84% identical residues as shown in Figure 2, and the two MutA's shared 93% identical residues. For MutB and MutC the similarity was

93% and 95%, respectively. An even higher similarity (99%) existed in MutP and -T between the two strains.

**Purification of mutacin I.** To biochemically characterize mutacin I, sufficient amount of starting material is required. Applicants' first attempt to isolate mutacin I from liquid culture failed because no mutacin I was produced in any of the liquid cultures that were tested. A stab culture on TH/agarose plate as described for mutacin III was then tried. Qi et al. (1999) *Appl. Environ. Microbiol.* **65:**652-658. Mutacin I was produced on such a plate, however the production level was still too low for satisfactory isolation. Based on the observation that mutacin I could be produced on all solid media plates regardless of the media composition, it was reasoned that the production of mutacin I may be regulated by a cell-density mediated control mechanism similar to quorum sensing. (Kleerebezem et al. (1997) *Mol. Microbiol.* **24:**895-904; Surette et al. (1999) *Proc. Natl. Acad. Sci. USA* **96:**1639-1644). Based on this rationale, a membrane transfer technique as described in Materials and Methods was employed, which resulted in a high level of mutacin I production.

Mutacin I was purified by reverse-phase HPLC as shown in Figure 3. The active fraction (fraction 6) from the first pass (see Figure 3A) was collected and subjected to a second round purification using a different buffer B and a different gradient (see Figure 3B). The active fractions (fractions 6 and 7) from the second pass were dried under vacuum and tested for purity by EIMS analysis. As shown in Figure 3C, mutacin I was purified to near

homogeneity as judged by the lack of significant background peaks in the MS chromatogram.

**Characterization of mutacin I by ethanethiol derivatization and MS analyses.** The molecular weight of mutacin I was measured by electrospray ionization mass spectrometry. The mass-to-charge ratio for the doubly-charged molecule was 1183, and that for the triply-charged molecule was 788 as shown in Figure 3C. Thus the measured molecule mass was 2364 Da. This value was in a good agreement with the calculated value of 2516 Da for the unmodified mutacin I minus six molecules of water (108 Da) and one molecule of carboxy residue (45 Da from decarboxylation at the C-terminal cycteine residue).

The primary sequence of mutacin I contained six serine residues and one threonine residue, all of which were potential sites for post-translational dehydration. To confirm that there were indeed six dehydrated residues in the mature mutacin I, an ethanethiol modification of mutacin I under alkaline conditions was performed. In this reaction, one molecule of ethanethiol could insert into the thioether bridge, resulting in a S-ethylcystein and a cystein, or it could insert into the double bound of a dehydrated serine or threonine to form a S-ethylcystein or a β-methyl-S-ethylcycteine. Meyer et al. (1994) *Anal. Biochem.* **223:**185-190; Novak et al. (1996) *Anal. Biochem.* **236:**358-360. Ethanethiol derivatization of lantibiotics has been used prior to sequencing of the other lantibiotic gallidermin and pep5 (Meyer et al. (1994) *Anal. Biochem.* **223:**185-190), and for determination of the number of dehydrated amino acid residues in mutacin II (Novak et al. (1996) *Anal. Biochem.* **236:**358-360). The

expected molecular mass of mutacin I after each addition of an ethanethiol molecule is listed in Table 1.

Table 1

Expected molecular masses of ethanethiol derivatives of mutacins I and III

| Mutacin | Expected mass (Da) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0* | 1 | 2 | 3 | 4 | 5 | 6 |
| I | 2,364 | 2,426 | 2,487 | 2,549 | 2,611 | 2,673 | 2,738 |
| III | 2,264 | 2,318 | 2,390 | 2,452 | 2,514 | 2,576 | 2,638 |

*Number of ethanethiol molecules added.

Quite surprisingly, none of the major peaks generated after ethanethiol modification of mutacin I had the expected molecular mass as shown in Figure 4A. A very small portion of the molecules showed a pass of 2736 Da (Peak 7), which could account for mutacin I plus six molecules of ethanethiol (2364 + 62x6); the result of the molecules were all much smaller than expected. With close inspection and calculations, the identity of the small molecules was determined. As shown in Figure 4B, it appeared that the majority of mutacin I molecules broke into two fragments after the addition of six molecules of ethanethiol. The larger fragment with a mass of 1791 Da was the N-terminal part from F-1 to N-16, and the smaller fragment (965 Da) was the C-terminal part from P-17 to C-24. This finding was of interest because the closely related mutacin III molecule remained intact after the same modification reaction under the same conditions as shown in Figure 4C.

**Peptide sequencing of unmodified and ethanethiol modified mutacin I.** Comparison of mutacin I and mutacin III revealed that mutacin I

had seven potential dehydration sites (six serines and one threonine), while mutacin III had six (four serines and two threonines). Interestingly, both mutacins had six ethanethiol additions after ethanethiol modification (see Figure 4A and 4C), suggesting that all serine or threonine residues in mutacin III were dehydrated. To determine which serine or threonine residue was not dehydrated in mutacin I, the purified mutacin I was subjected to peptide sequencing by Edman degradation. With native mutacin I, Edman degradation was blocked after the first F residue, suggesting that the second serine residue is dehydrated. Dehydrated amino acids were shown to block Edman degradation in other lantibiotics. Gross et al. (1971) *J. Am. Chem. Soc.* **93:**4634-4635; Mota-Meira et al. (1997) *FEBS Lett.* **410:**275-279; Novak et al. (1994) *J. Bacteriol.* **176:**4316-4320.

To get a complete sequence of mutacin I, the ethanethiol-derivatized mutacin I had to be used. Ethanethiol-derivatization of lantibiotics was shown to allow Edman degradation to proceed through the dehydrated serine and threonine residues and thioether bridges in other lantibiotics. Meyer et al. (1994) *Anal. Biochem.* **223:**185-190; Mota-Meira et al. (1997) *FEBS Lett.* **410:**275-279. Since the majority of mutacin I molecules was broken into two fragments (see Figure 4) during ethanethiol modification, the C-terminal fragment had to be eliminated to solve the problem of having two N-termini in the reaction mixture. After several trials, the C-terminal fragment was eliminated by washing the reaction mixture with 30% acetonitrile. The pellet fraction after 30% acetonitrile wash contained mostly the full-length modified

mutacin I and the N-terminal fragment. Sequencing of the pellet fraction revealed the following sequence: $F_1$-$SEC_2$-$SEC_3$-$L_4$-$SEC_5$-$L_6$-$SEC_7$-$SEC_8$-$L_9$-$G_{10}$-$SEC_{11}$-$T_{12}$-$G_{13}$-$V_{14}$-$K_{15}$-$N_{16}$-$P_{17}$-$SEC_{18}$-$F_{19}$-$N_{20}$-$SEC_{21}$-$Y_{22}$-$SEC_{23}$. S-ethylcysteine (SEC) was the product of ethanethiol insertion into the double bond of dehydrated serine, or the thioether bridge in lanthionine. The results revealed that all six serine residues in the mutacin I molecule were dehydrated, and that T-12 remained as a nondehydrated residue. In addition, a closer look at the HPLC chromatogram of the sequencing reaction of mutacin I revealed minor peaks in the sequence of P-x-F-N-x-Y. This sequence correlated with the C-terminal fragment of mutacin I: $P_{17}$-$S_{18}$-$F_{19}$-$N_{20}$-$S_{21}$-$Y_{22}$-$C_{23}$-$C_{24}$. This result corroborated the previous assignment for the two peptide fragments generated during ethanethiol modification as shown in Figure 4B.

The mutacin I biosynthesis genes from the group I strain of *S. mutans* CH43 were cloned and sequenced. DNA and protein sequence analysis revealed that mutacin I and mutacin III are highly homologous to each other, likely arising from a common gene ancestor. Mutacin I was produced by a membrane transfer technique and purified to homogeneity by reverse phase HPLC. The mature mutacin I is twenty-four amino acids in size with a molecular weight of 2364 Da. Ethanethiol modification of mutacin I revealed that it contains six dehydrated amino acids. Sequencing of the native and ethanethiol-derivatized mutacin I by Edman degradation demonstrated that mutacin I is encoded by *mutA*, and that the six serine residues in the primary

sequence of mutacin I are dehydrated, four of which are possibly involved with thioether bridge formation. Comparison of the primary sequence of mutacin I with that of mutacin III and epidermin suggests that mutacin I likely possesses the same bridging pattern as epidermin.

A closer inspection of the differences between the homologous genes of mutacin I and mutacin III revealed that they are not all distributed evenly. For MutR, -D, -P, and -T, the homology is over 99% between the two mutacins, while for MutA, -A', -B, and -C, the similarity varies from 87 to 95%. The distribution of the variations within a protein is not even either. For example, in MutA, the leader peptide region was identical between the two mutacins. However, the mature peptide region differed by 37.5% (Fig. 2). More interestingly, the sequence of the mature mutacin III is closer to that of epidermin (77% similarity) than to mutacin I (62.5% similarity), while the sequence of the leader peptide of mutacin III and epidermin are dramatically different as seen in Figure 2. For MutB, -C, -D, -P, and -T proteins, mutacin I and mutacin III are closer to each other than to epidermin.

The biosynthesis of lantibiotics involves several posttranslational modification steps. Chakicherla et al. (1995) *J. Biol. Chem.* **270:**23533-23539; de Vos et al. (1995) *Molecular Microbiol.* **17:**427-437; Sahl et al. (1998) *Annu. Rev. Microbiol.* **52:**41-79. The first step is the translation of the structural gene message into a prepropeptide. The prepropeptide is then modified by dehydration of serine and threonine residues, and formation of thioether bridges between cysteine and the dehydrated amino acid residues. The

prepeptide is then translocated across the cell membrane, where the leader peptide is cleaved off and the mature peptide released to the outside medium.

One advantage of lantibiotics over classical antibiotics is its gene-encoded nature, which means that lantibiotics can be altered with ease by manipulating the structural genes through mutagenesis. In reality, however, the number of mutations that can be made is limited because the production of active lantibiotics depends on correct post-translational modification and processing.

Mutacin I and mutacin III are closely related to each other at both the nucleotide and amino acid levels. Comparison of the mature peptide sequence of mutacin I and mutacin III suggests that they may also have the same pattern of thioether bridge formation. Despite all the similarities, some important differences exist between the two mutacins. For example, ethanethiol modification of mutacin I broke the molecule into two fragments between N-16 and P-17 as shown in Figure 4B, while the same reaction did not affect the integrity of mutacin III as shown in Figure 4C. Comparison of the two mutacins revealed that the major difference is at the linker region (T-12 to P-17), where mutacin I has the sequence T-G-V-K-N-P, and mutacin III has the sequence A-R-T-G as shown in Figure 2A. These different amino acid residues, according to the statistical figures of Creighton (Creighton, p. 235, in (ed.) Proteins: Structures and molecular principles, W.H. Freeman and Company, New York), have different tendencies in forming different secondary structures in proteins. For example, N-16 and P-17 in mutacin I are

more likely to be involved in forming β-turns, while A-12 in mutacin III is more likely to participate in α-helix formation (Stryer, p. 37, in (ed.) Biochemistry, W.H. Freeman and Company, Biochemistry, New York). More importantly, N-16 and P-17 are absent in mutacin III.

In accordance with the possible difference in secondary and tertiary structures, mutacin I and mutacin III have different hydrophobicity and antimicrobial activity. In reverse-phase HPLC analysis, mutacin I is eluted at a higher acetonitrile concentration than mutacin III, suggesting that it is more hydrophobic than mutacin III. In antimicrobial spectrum assays with a limited set of pathogens, mutacin III is more potent than mutacin I against *Staphylococcus aureus* and *Staphylococcus epidermidis*, while both mutacins have equal activities against other pathogens such as enterococci, pneumococci, and Group A streptococci.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended

as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.